Paroxysmal Nocturnal Hemoglobinuria: The Role of Properdin in the Diagnostic Thrombin Test

By William H. Crosby and Naomi R. Benjamin

The red blood cells of patients with paroxysmal nocturnal hemoglobinuria (PNH) possess an apparently unique susceptibility to the hemolytic activity of certain naturally occurring plasma factors. Hemolysis occurs readily in vitro and the reaction is intensified when the incubating mixture has been made slightly acid. This phenomenon is the basis of a standard diagnostic test for the disease, the Ham acid-hemolysis test. Several years ago it was noted that increased hemolysis in patients with PNH was sometimes associated with increased activity of the blood coagulation mechanism. A relationship between PNH hemolysis and the coagulation system was also demonstrated in vitro: hemolysis was increased by adding to the Ham test small amounts of thrombin or thromboplastin. This phenomenon has proved diagnostically useful in the "thrombin test" for PNH. It has been suggested that thrombin causes this increased hemolysis by destroying or blocking an inhibitor of the PNH hemolytic system.

More recently it has been announced that the PNH hemolytic system depends upon the bactericidal globulin, properdin. This protein has been prepared in a relatively pure form, and it has been demonstrated that properdin plus Mg++, together with other plasma factors as yet unidentified, can hemolyze PNH cells in acidified serum. It was also found that commercially available preparations of thrombin, of the sort used for the "thrombin test," are contaminated with properdin. On the basis of these data, it was suggested that the effect of thrombin on the PNH hemolytic system is due to the presence of the contaminating properdin.

In the present report are described some further observations bearing upon this point.

Materials and Methods

PNH red cells were obtained from three patients with classical history and signs of the disease. The cells were prepared by washing them three times in isotonic saline solution. Serum was obtained from normal donors of compatible blood groups. It was prepared by centrifuging unclotted blood in siliconized tubes, transferring the plasma to glass tubes and squeezing out the fibrin clot when it had formed. Serum for the acid-hemolysis test was acidified just before use by adding 5 per cent by volume of N/3 HCl; the pH of such serum was approximately 6.8. Thrombin solution was prepared by dissolving 5000 units of powdered bovine thrombin (Thrombin Topical, U.S.P., Parke, Davis & Co.) in 10 ml. of serum. The amount of magnesium in the powdered thrombin (0.14 mg. per 5000 units) is negligible when the material is used as it was in the present experiments. Properdin was removed from serum and the thrombin-serum mixtures by incubating them for two hours at 17 C. with activated zymosan, 2 mg. per ml. of serum. The zymosan was...
continually resuspended and it was replaced by fresh zymosan at the end of one hour. At the end of the second hour the zymosan was again replaced and incubation was continued for 20 minutes with the temperature increased to 37 C. Prior to treatment with zymosan the presence of properdin was demonstrated in the serum-thrombin preparation by means of the RP control titration technic of Pillemer. After incubation with zymosan no properdin activity was demonstrable; it was also possible to elute properdin from the zymosan which had been used for the incubation. Even though the presence of thrombin interfered with the accurate assay of properdin it did not interfere with its removal from the thrombin-serum mixture by incubation with zymosan. Activity against fibrinogen of the thrombin-serum mixtures was essentially unaltered after incubation. Zymosan-treated serums and the zymosan-treated serum-thrombin mixtures were acidified to pH 6.8 and tested against PNH red cells. Both were completely inactive, and mixtures of the two were also devoid of PNH hemolytic activity.

Solutions of thrombin in serum, 500 units per ml., were heated at 56 C. for 30 minutes and again at 56 C. for 60 minutes, sufficient to inactivate completely properdin in serum. The activity of these thrombin solutions tested against fibrinogen was greatly diminished after 30 minutes and absent after 60 minutes.

Experimental

Washed, packed red cells were added, 5 per cent by volume, to 5 ml. of cold, acidified serum and mixed. The suspension was immediately divided into 5 aliquots, placing each in a test tube which contained 0.1 ml. of an additive to be tested. In two of the tubes the additive was zymosan-treated serum to which no thrombin had been added. One of the two was centrifuged immediately to serve as an unincubated control. The additive in the third tube was the serum-thrombin solution which had not been treated with zymosan and in the fourth tube was the zymosan-treated solution of thrombin. The fifth tube contained unmodified serum. The tubes were shaken and placed in a water bath at 37 C. for 15 minutes. They were removed, shaken again and centrifuged. The serum was removed and the concentration of hemoglobin was measured.

In a similar experiment the effect of the heated thrombin solution was compared with that of the same solution unheated.

Results

In the first experiment it was found that the serum-thrombin mixture increased hemolysis when it was added to the PNH cell suspensions (table 1). Previous treatment of the mixture with zymosan diminished only slightly its ability to alter the intensity of the reaction. Similar results were obtained when the "thrombin test" was performed using thrombin preparations which had been heated to destroy properdin (table 2). Zymosan-treated solutions of thrombin in serum were passed through cation-exchange resins to remove all Mg++. Such preparations retained their activity in the acid-hemolysis test, as did solutions of thrombin that were dialyzed against saline to remove Mg++. The titrations were performed by Mr. Andre J. Toussaint, Department of Serology, Walter Reed Army Institute of Research.

Discussion

If incubation with zymosan or heating at 56 C. is able to remove or inactivate the properdin in serum, as claimed, then one may conclude from the present experiments that the properdin which contaminates thrombin preparations has little to do with the results of the "thrombin test."
CREASING HEMOLYSIS WHICH IS PRODUCED BY THE ADDITION OF THROMBIN TO THE PNH HEMOLYTIC SYSTEM IN VITRO MAY BE DUE TO SOME ACTION OF THROMBIN ITSELF OR TO A DEGRADATION PRODUCT OF THROMBIN OR TO A CONTAMINATION OTHER THAN PROPERDIN. IT IS SUSPECTED, HOWEVER, THAT THE COAGULATION MECHANISM IS SOMEHOW INVOLVED BECAUSE THROMBOPLASTIN HAS THE SAME EFFECT AS THROMBIN ON THE ACID-HEMOLYSIS TEST, AND THE ADDITION OF PLATELETS DURING THE CLOTTING OF PLATELET-POOR PLASMA CAN GREATLY INCREASE THE HEMOLYTIC ACTIVITY OF THE SERUM THAT IS FORMED.² BOTH PLATELETS AND THROMBOPLASTIN ARE DEVOID OF PROPERDIN ACTIVITY.⁵

IT IS UNLIKELY THAT THE FIBRINOGEN-CLOTTING EFFECT OF THROMBIN IS INVOLVED IN THE THROMBIN TEST. THIS ACTIVITY WAS LOST WHEN THE THROMBIN-SERUM PREPARATIONS WERE HEATED AT 56 C. FOR 30 OR 60 MINUTES TO DESTROY THE PROPERDIN. YET THIS HEATING DID NOT SUBSTANTIALLY INTERFERE WITH THE ACTIVITY OF THE MATERIAL IN THE THROMBIN TEST (TABLE 2). HOWEVER, THE HEATING OF SUCH THROMBIN PREPARATIONS DOES NOT ENTIRELY DESTROY THEIR ACTIVITY IN THE COAGULATION SYSTEM; ALTHOUGH THEY DO NOT CLOT FIBRINOGEN THEY ARE CAPABLE OF SHORTENING THE CLOTTING TIME OF RECALCIFIED HUMAN PLASMA.⁶ IT HAS BEEN SUGGESTED THAT THIS EFFECT ON THE RECALCIFICATION TIME MAY BE RELATED TO AN INCREASED RATE OF GENERATION OF THROMBOPLASTIN.

THE ROLE OF PROPERDIN IN THE ACID-HEMOLYSIS TEST CANNOT BE GAINSAYED, BUT THE ADDITION OF THROMBIN TO THE TEST DOES NOT MERELY PROVIDE MORE PROPERDIN. IT SOMEHOW IMPROVES THE EFFECTIVENESS OF THE HEMOLYTIC FACTORS ALREADY PRESENT.

SUMMARY

1. The “thrombin test” for PNH consists of adding thrombin to the standard acid-hemolysis test, thereby increasing hemolysis of PNH red cells. It has
been suggested that the effect of the thrombin solution is not due to involvement of the coagulation system but to contamination of the thrombin by properdin, a globulin which is essential in the acid-hemolysis reaction of PNH.

2. In the present experiments the thrombin test was positive using preparations of thrombin which were free of properdin activity.

3. Thromboplastic materials devoid of properdin activity may be substituted for thrombin in the thrombin test.

4. It is concluded that the properdin which contaminates the commercially available thrombin preparations has little to do with the results of the thrombin test for PNH.

SUMMARIO IN INTERLINGUA

1. Le “test a thrombina” pro nocturne hemoglobinuria paroxysmal consiste de un addition de thrombina al test standard de hemolyse acide. Isto resulta in un augmento del hemolyse de erythrocytos de nocturne hemoglobinuria paroxysmal. Il ha essite proponite que le effecto del solution de thrombina non debe esser explicate per un affection del systema de coagulation sed per le contamination de properdina per thrombina. Iste conception se basa super le facto que properdina es un globulina que es indispensabile in le reaction de hemolyse acide in nocturne hemoglobinuria paroxysmal.

2. In le presente experimentos le test a thrombina esseva positive in despecto del facto que preparatos de thrombina esseva usate que esseva libere de activitate properdinic.

3. Materiales thromboplastic disproviste de activitate de properdinic pote esser usate in loco de thrombina in le test a thrombina.

4. Es concludite que le properdinic que contamina le commercialmente disponible preparatos de thrombina ha pauco a facer con le resultatos del test a thrombina pro nocturne hemoglobinuria paroxysmal.

REFERENCES


Paroxysmal Nocturnal Hemoglobinuria: The Role of Properdin in the Diagnostic Thrombin Test

WILLIAM H. CROSBY and NAOMI R. BENJAMIN